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Abstract

This study was conducted to investigate the present of phytoplasma in coconut palms showing symptoms of Awka wilt disease or lethal yellowing disease (LYD) in Nigeria and determine if there is any geographical differentiation between the phytoplasma strains. Stem samples were collected from symptomatic coconut palms in five States namely; Anambra, Abia, Imo (in the south-east zone on Nigeria) and Edo and Akwa Ibom (in south-south zone of the coconut belt of Nigeria). DNA was extracted from the samples using cethyl trimethyl ammonium bromide (CTAB) protocol and the 16SrDNA was amplified by polymerase chain reaction (PCR) technique using the universal P1/P7 primers. The PCR products were sequenced and compared with each other. Five samples (NG12-30; NG12-36; NG12-37; NG12-43; NG12-44) out of the sixteen samples examined, yielded phytoplasma-specific 1,756 bp fragments when amplified with P1/P7 primers. A homology of 100% was observed between the sequences of the five samples based on the known conserved 16SrDNA sequences. The result of this study showed that phytoplasma was present in coconut palms that exhibited symptoms of Awka wilt disease and the phytoplasmas in the south-east and south-south zone of the country were the same strain. A similar investigation for symptomatic coconut from the western zone of the country is anticipated.

Keywords: Phytoplasma, Awka wilt, Coconut, Polymerase Chain Reaction

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Introduction

Lethal yellowing disease (LYD), known as Awka wilt disease in Nigeria, is a highly destructive and most serious disease of coconut. The disease was first reported in Awka district of Eastern Nigeria in 1917 (Johnson, 1918). The symptoms of the disease commences with premature dropping of nuts and blackening of inflorescence followed by yellowing of fronds, desiccation and finally, death of the palm occurs within 9 to 12 months with the affected palms looking like electric poles (Osagie and Asemota, 1995). The disease can wipe out an entire coconut plantation as in the case of the coconut seed gardens of Nigerian Institute For Oil Palm Research (NIFOR) located in the Main Station (Plate 1a) and Ubiaja Experimental Station in Edo State (Plate 1b) where the coconut palms are at the point of total elimination by the disease. Awka wilt is a phytoplasma disease (Tymon, 1993) similar to Kribi disease in Cameroon (Dollet et al., 1977), Kaincope disease in Togo (Nienhaus and Steiner, (1976), Cape St Paul Wilt (CSPW) in Ghana (Johnson and Harries, 1976; Nipal et al., 2007) and Lethal Disease (LD) in Tanzania (Shuiling et al., 1981). Studies have shown that the predominant West African Tall (WAT) coconuts in Nigeria are highly susceptible to the disease whereas, the exotic Malayan Green Dwarf (MGD) coconuts were relatively tolerant in a field trial at Urhokuosa village, near Benin.

Consequently, the MGD coconut was recommended for controlling the disease (Osagie et al., 1998). Unfortunately, the tolerance of this exotic palm was not durable as over 50% of these MGD coconut palms were destroyed by the disease in NIFOR coconut seed garden where 98% of the WAT coconut palms were also killed by the disease after 13 years (Osagie and Ojomo, 2011). Also the MGD x VTT hybrid coconuts that were tolerant to Cape St Paul wilt in Ghana were destroyed by the disease when planted in Nigeria (Osagie and Ojomo, 2010 Unpublished). These observations tended to raise questions about the durability of coconut's tolerance to the disease or the nature of the LYD phytoplasma. Moreover, there is a disagreement among Research Scientists in NIFOR whether the typical symptoms of Awka wilt disease on coconut palms in different parts of the country were actually caused by phytoplasma. According to Nejat and Vadamalai (2010), the detection and identification of phytoplasma is a requirement for accurate LYD diagnosis.

The objectives of this study are to detect the presence of phytoplasma in coconut palms showing symptoms of Awka wilt disease in different parts of Nigeria and determine whether the strains of the phytoplasma detected are the same or different.

Materials and Methods

Samples were collected from the stem of coconut palms showing symptoms of Awka wilt disease i.e. premature nut fall, blackening of inflorescence and yellowing of the fronds (Plate 2a). The age of the sampled coconut trees (mostly WAT cultivars) ranged between 15 and 60 years. The samples were collected from 5 States and 16 locations in south-east and south-south zone of Nigeria (Table 1) in August 2012 during the raining season. A motorized drill attached with flame sterilized 10 mm drill bit, was used for collecting the samples as described by Pilet et al. (2011). The point of collection on the stem was first surface-sterilized by spraying with bleach and after 2 to 3 minutes, it was rinsed with clean water and a portion of the bark measuring about 10 cm x 10 cm x 2 cm was carefully sliced off with a sharp flame-sterilized cutlass. The drill bit was vertically positioned at that point and the drill was operated such that the saw dust coming out was collected with 25 ml sterile urine sample bottles placed below the drill (Plate 2b). The sample bottles contained 7.5 gm silica gel to absorb moisture, keep the samples dry and prevent microbial growth. After collection, the samples were initially stored at room temperature before being taken to Montpellier, France and stored at -80^{o}C prior to DNA extraction.

DNA extraction and PCR assay were carried out in the Institut de Recherche pour le Developpement (IRD) laboratory in Montpellier, France using cetyl trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990) with some modifications. 500 mg of the coconut saw dust was grounded at room temperature with 4 ml of CTAB extraction buffer (pH 8.0) composed of Tris, 1.21 g; CTAB, 3 g; NaCl, 8.1 g; EDTA, 0.8 g; and 100 ml ultra-pure water to which a pinch of poly venyl polypyrrolidine (PVPP) was added. 750 µl of the homogenized tissue was taken in 2 ml tubes and centrifuged at 13200 rpm for 5 min. 1ml of the supernatant was transferred to another 2 ml centrifuge tube and 2 µl of RNAse (100 mg/ml) was added. The tube was vortexed, left for at least 5 min on the bench and incubated at 65^{o}C for 20 min with occasional mixing during incubation. 1 ml of chloroform/ isoamyl alcohol (24: 1) was added to the tube and after shaking vigorously, the tube was centrifuged at 13200 rpm for 15 min. The aqueous phase was carefully pipetted and transferred to a 1.5 ml centrifuge tube ensuring that no chloroform was taken along. The DNA was precipitated with 750 µl of cold (-20^{o}C) isopropanol and pelleted by centrifugation (13200 rpm for 15 min, 4^{o}C). The pellet was washed with 1 ml chilled 70% ethanol, centrifuged at13200 rpm for 10 min and the supernatant was discarded. The DNA was dried in a vacuum centrifuge dryer and re-suspended in 50 µl of ultra-pure water (or EB buffer for long preservation) after which the concentration was determined by measuring the optical density at 260 nm.

The phytoplasma 16S rDNA was amplified by the polymerase chain reaction (PCR) protocol using the universal primer pair P1 (5^{1}- AAG AGT TTG ACT CTG GCT CAG GAT T-3^{1}) and P7 (5^{1}- CGT CCT TCA TCG GCT CTT -3^{1}) derived from highly conserved ribosomal sequences (Deng and Hiruki, 1991; Smart *et al*., 1996). The DNA samples extracted from symptomatic coconut palms were used as template for PCR analysis. For the P1/P7 PCR assay, the 25µl of reaction mixture contained 1.5µl of extracted DNA, 1.0µl of each primer, 0.5µl of dNTPs, 0.25µl of Taq polymerase, 2.5µl of 10x PCR buffer and 18.25 µl of Ultra-Pure H_2O. The PCR reaction conditions were conducted at 94^{o}C for 5 minutes, followed by 35 cycles of 94^{o}C denaturation temperature for 30 seconds, 50^{o}C annealing for 1 minute and 72^{o}C extension for 2 minutes. Post PCR extension was carried out at 72^{o}C for 7 minutes and finally the material was stored at 4^{o}C.

After amplification, 3.0 µl aliquot from each PCR products was electrophoresed in 1.0% (w/v) horizontal agarose gel bed in Tris borate EDTA (TBE) buffer at 100 volt and 50 mA current for 1 hr 30 min. The gel was visualized after staining with ethidium bromide (EtBr) under UV trans-illumination. The P1/P7 PCR products that showed a clear and strong

amplification were sequenced by Beckman Coulter Genomics (UK). The sequences were aligned by using Clustal W and analyzed with DnaDist under Bioedit version 7.0.9.0 programme software.

Results and Discussion

Coconut palms were surveyed in five States in the coconut belt of Nigeria and stem samples were collected from the palms showing typical symptoms of Awka wilt disease, namely premature nut fall, blackening of inflorescence and yellowing of fronds. A total of 16 samples (3 from Anambra State, 3 from Abia State, 3 from Akwa Ibom State, 4 from Edo State and 3 from Imo State) were collected from mostly the WAT coconut cultivars (Table 1). The results showed that out of the 16 stem samples collected from coconut palms showing symptoms of Awka wilt disease in five States of Nigeria, after DNA extraction, 5 samples (NG 12-030, NG 12-036, NG 12-037, NG 12-043 and NG 12-044) yielded phytoplasma-specific 1756 bp products when amplified by PCR using P1/P7 primer pair (Figure 1). These samples were collected from Abak in Akwa Ibom State, NIFOR in Edo State, Usen in Edo State and Ekwulobia in Anambra State. This confirmed the presence of LYD phytoplasma in the samples. Sequencing results obtained from the ribosomal protein gene 16S rRNA by Beckman Coulter Genomics in UK showed that the phytoplasma in the five samples showed 100% homology (Figure. 2).

The result of this study showed that phytoplasma was detected in coconut palms showing symptoms of Awka wilt disease by using PCR technique after DNA extraction. According to Smart et al. (1996), PCR is the most versatile tool for detecting phytoplasma in their plant and insect hosts. Five out of the sixteen samples collected from symptomatic coconut palms in five States of Nigeria were positive for presence of phytoplasma. This finding settles the doubt regarding phytoplasma association with Awka wilt disease because according to Nejat and Vadamalai (2010), detection and identification of phytoplasma is essential for LYD diagnosis. It will be recalled that Awka wilt disease formally called Bronze leaf wilt was first reported in Nigeria in 1917 (Johnson, 1918) but it was not until 1993 (75 years later), that the etiology of the disease was reported to be a Mycoplasma-like organism (MLO), now phytoplasma by Thymon (I993). After that study by Thymon (1993), no other diagnostic studies on etiology of Awka wilt disease have been conducted apart from the present study. Sequencing of the PCR products of the five positive samples showed 100% homology, indicating that the phytoplasmas were the same strain. This finding is not only important etiologically, but also very fundamental for the development of a proper disease management. From this finding, a tolerant coconut cultivar in one location of sample collection can hopefully be planted in any of the other 15 locations in Nigeria to control the disease. A similar study carried out in Ghana based on other genes, reported the occurrence of genetic differentiation in the phytoplasma strains causing Cape St Paul Wilt disease of coconut in that country (Pilet et al., 2011).

Conclusion and Recommendations

This study detected phytoplasma in five out of the sixteen samples collected from the stem of symptomatic coconuts possibly because some of the samples were burnt due to the heat generated by the process of drilling during sample collection. However, other studies have found that phytoplasma detection from samples depended on a number of factors such as the plant part used for DNA extraction (Kollar et al., 1990), and uneven distribution of phytoplasma in the phloem vessels of infected plants (Davies and Lee, 1993; Jones et al., 1999). Moreover, Braun and Sinclair (1982) reported that in woody plants, the persistence of phytoplasma in aerial parts of the host plants depended on the seasonal state of the phloem as well as environmental and climatic factors (Nejat and Vadamalai, 2010). Further investigation for phytoplsama in symptomatic coconut palms in the western zone of the country particularly Lagos state in which the major coconut grove resides, is anticipated.

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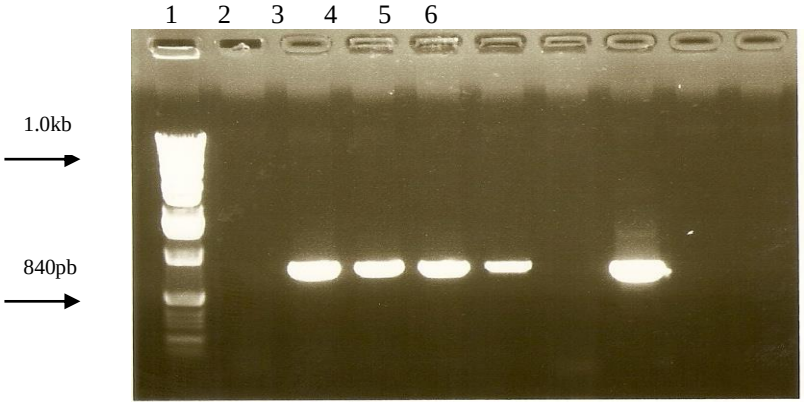
Table

Table 1: Sample identification number and places of collection

Sample Number	Place of Collection	
	State	**Town/Village**
NG12-022	Abia	Isiala-Oboro
NG12-023	Abia	Isiala-Oboro
NG12-024	Abia	Oboro-Ikwano
NG12-026	Akwa-Ibom	Ikot-Ekpene
NG12-029	Akwa-Ibom	Abak
NG12-030	Akwa-Ibom	Abak
NG12-036	Edo	NIFOR
NG12-037	Edo	NIFOR
NG12-041	Edo	Ubiaja
NG12-043	Edo	Usen
NG12-044	Anambra	Ekwulobia
NG12-045	Anambra	Uga
NG12-046	Anambra	Oba
NG12-048	Imo	Nkwerre
NG12-049	Imo	Mbano
NG12-050	Imo	Umuelemai

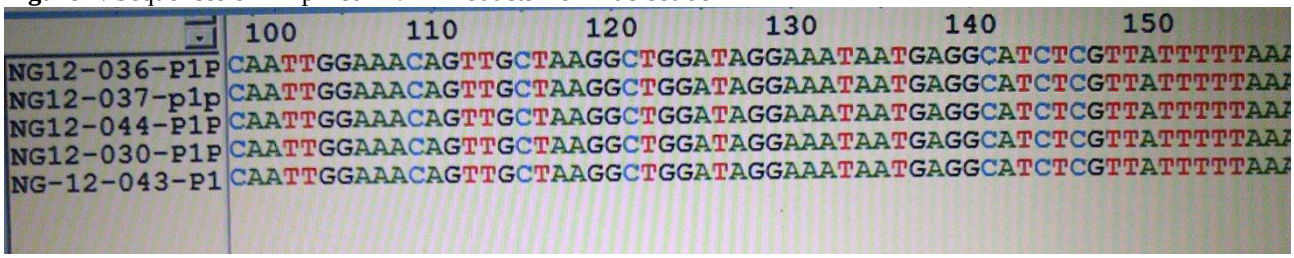
Figures

Figure 1: PCR Amplified 16S rRNA with P1/P7 primer pair.



Lane 1, NG 12–036: Lane 2, NG12 -037: Lane 3: NG12-043: Lane 4, NG12-044: Lane 5, -ve control (water). Lane 6, +ve control (Phyllodie).

Figure 2: Sequences of Amplfied P1/P7 Products from nucleotide



positions 100 to 150 for the five samples (NG12-036, NG12-037, NG12-044, NG12-030 & NG12-043).

Plates

Plate 1: Coconut Palms Affected by Awka Wilt Disease in Coconut Seed Gardens at NIFOR (Plate 1a) and Ubiaja (Plate 1b).

Plate 1a

Plate 1b

Plate 2: Coconut palms showing symptoms of Awka Wilt (Plate 2a) and sample collection with motorized drill (Plate 2b).

Plate 2a

Plate 2b